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13. ABSTRACT (Maximum 200 Words)
Receptor tyrosine kinases of the erbB family play pivotal roles in growth and differentiation and aberrant activation of these receptors is associated with human cancers. In particular, ErbB-2 dysfunciton has been linked to about 30% of breast cancers with poor prognosis. Correspondingly, great efforts are being made to develop therapies that target ErbB pathways. ErbB-2 is activated by the neuregulins in heterodimers with the neuregulin receptors ErbB-3 and ErbB-4. An antagonistic neuregulin that down regulates ErbB signaling could function as an anti-tumor agent. The purpose here is to develop such a factor. In previous work, the Drosophila system was used to demonstrate that an antagonistic neuregulin-like factor could be made by deleting the EGF domain or by insertion of the EGF domain from a natural inhibitor. In this project, a vertebrate neuregulin-1 with an EGF domain deletion (NRGΔEGF) and a factor with the EFG domain from the inhibitor (NRG::Aos-EGF) were made. The activity of the factors was tested in transgenic mice by examining heart defects which are characteristic of neuregulin defects. No defects were observed. The results suggest the factors are unlikely to be useful in regulating NRG function in the breast.

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Introduction

Receptor tyrosine kinases (RTKs) of the erbB family play pivotal roles in growth and differentiation during normal development. However, aberrant activation of these receptors is associated with a significant number and variety of human cancers. In particular, erbB-2 dysfunction has been linked to about 30% of breast cancers and these have a poor prognosis. Correspondingly, great efforts are being made to develop therapies that target erbB pathways. The purpose of this work is to develop a vertebrate neuregulin antagonist that has potential use as an anti-tumor agent in some breast cancers that involve erbB-2 dysfunction. The rationale for the proposed work is based on a novel finding in the fly system which shows deletion of the EGF domain, or insertion of the EGF domain from a natural inhibitor, converts the fly neuregulin, Vein, into an antagonist [1](Fig. 1). Similar modifications were made in a vertebrate neuregulin and the ability of the factors to act as inhibitors has been tested *in vitro* and *in vivo.*

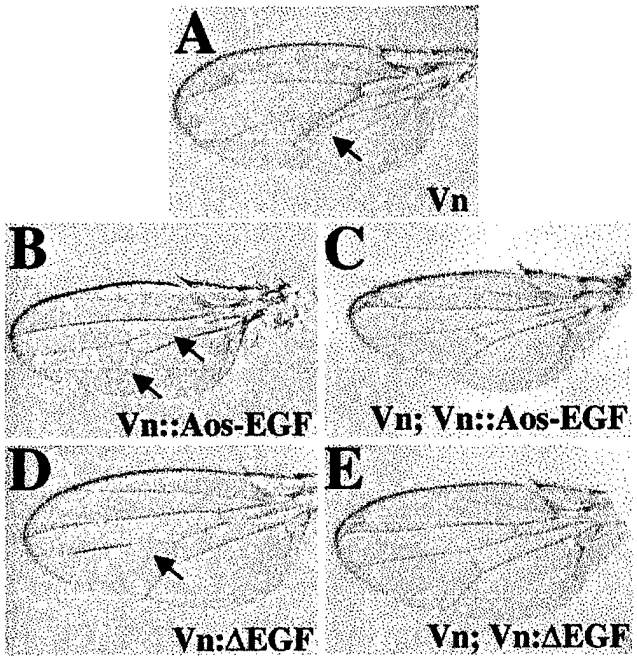

Figure 1. Vn::Aos-EGF is a more potent inhibitor than Vn:ΔEGF. A) Ectopic expression of the erbB agonist Vn causes extra veins to form indicating the EGF receptor has been activated (arrow). B) Expression of the inhibitor Vn::Aos EGF causes a loss of vein phenotype characteristic of EGF receptor inhibition (arrows) and a reduction in wing size. C) Co-expression of Vn and Vn::Aos EGF causes suppression of the extra vein phenotype, vein loss and a reduction in wing size. D) Expression of the inhibitor Vn:ΔEGF causes a loss of vein phenotype characteristic of EGF receptor inhibition (arrow) but wing size is normal. E) Co-expression of Vn and Vn:ΔEGF causes suppression of the extra vein phenotype. The extent of EGFR suppression is more pronounced following expression of Vn::Aos EGF where wing size is also affected (compare B with D and C with E).

Body

Task 1. To test the function of NRG-1: ΔEGF in cell culture.

1 a. Creation of an EGF deletion in NRG-1B

Our work in the fly system showed that deletion of the EGF domain from the fly neuregulin converted it into an erbB antagonist (Fig. 1). The goal here was to create a similar change in a vertebrate neuregulin. The NRGΔEGF construct was made as planned and we decided to also make a construct that has the EGF domain from the fly inhibitor called Argos

(Aos) (NRG::Aos-EGF). Our work in the fly system suggested this may be a more powerful inhibitor than the EGF deletion factor [1](Fig. 1).

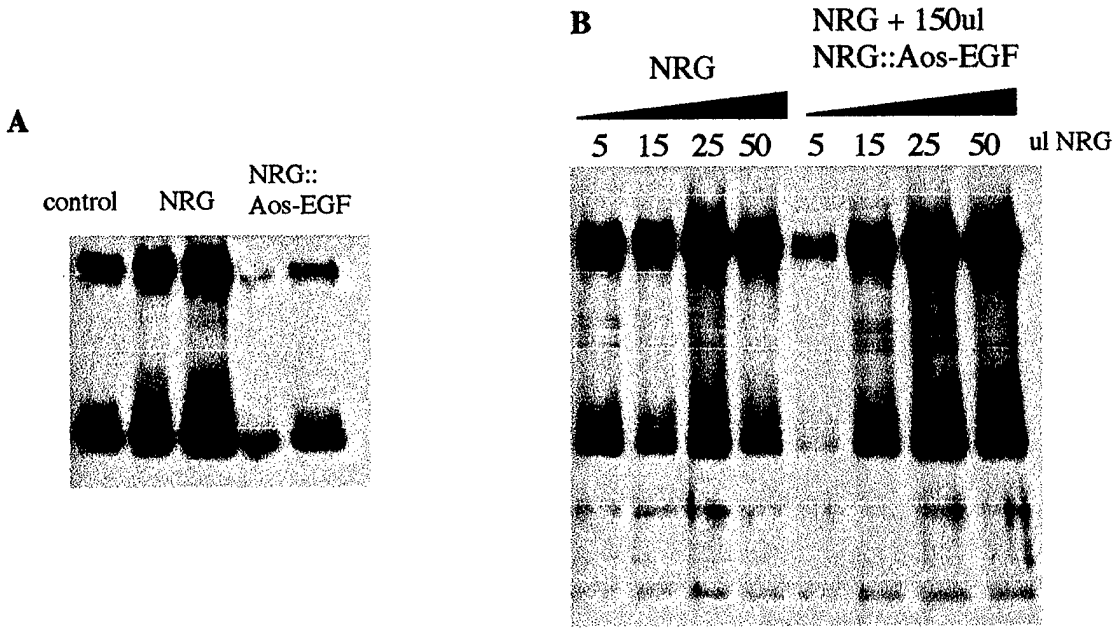

Figure 2 Inhibitory effects of NRG::Aos-EGF. ErbB4 expressing cells were exposed to control and conditioned media. The receptor was immunoprecipitated and the level of receptor phosphorylation was determined by blotting with anti-phosphotyrosine antibodies. A. The control shows the basal level of erbB4 phosphorylation (50ul of control medium was added to the cells). erbB4 phosphorylation increases upon stimulation with the agonist NRG (25ul and 50ul of NRG conditioned media were added to the cells). Addition of NRG::Aos-EGF reduced erbB4 phosphorylation below the control level (25ul and 50ul of NRG::Aos-EGF conditioned media were added to the cells). B. erbB4 expressing cells were exposed to increasing concentration of NRG (left lanes) or increasing concentrations of NRG following a pretreatment with NRG::Aos-EGF (left lanes). The level of erbB4 phosphorylation was determined. Pretreatment with NRG::Aos-EGF reduced receptor phosphorylation at the lowest concentration of NRG.

1 b. Testing effect of NRG-1: ΔEGF and NRG::Aos-EGF on NRG-1-activation of erbB receptors in mouse cells

We made large-scale preparations of DNA corresponding to the two factors (NRG-1ΔEGF and NRG-1::Aos-EGF) and sent these to our collaborator Dr. Stern at Yale medical school. Dr. Stern and colleagues transfected COS-7 cells with the DNA constructs and produced conditioned media containing the secreted factors. The activity of the factors was assayed in tissue culture cells expressing the receptor ErbB4. In this experiment the ability of the mutant factors to inhibit activation by native neuregulin was tested. Mouse cells expressing ErbB4 were treated with neuregulin or neuregulin in conjunction with a mutant factor. The receptor was immunoprecipitated and analyzed by western blotting with an anti-phosphotyrosine antibody. The level of phosphorylation is a measure of receptor activation. Most experiments were done with NRG::Aos-EGF. Unlike the parental factor NRG, NRG::Aos-EGF did not behave as an activator and could in fact reduce erbB4 signaling below baseline (the level of signal when control medium was added) (Fig. 2A). Furthermore, NRG::Aos-EGF was able to reduce

activation of the receptor by NRG (Fig. 2B). These results were consistent with the factor acting as an inhibitor and we therefore began testing in cancer cells.

1 c. Testing effect of NRG-1: ΔEGF on human breast cancer cell lines

In these experiments we tested the ability of the mutant neuregulins to block signaling through erbB receptors in human breast cancer cells. The cancer cell lines tested in these experiments were MDA-MB-453, MDA-MB-468, MCF7, and MDA-MB-175-VII. The control neuregulin (pHM1-NRG) and the mutant factors (pHM1-NRGΔEGF, pHM1-NRG::Aos-EGF) were produced by transfecting COS-7 cells. Conditioned media were collected and concentrated to 1/10 volume using Ultrafree centrifuge columns (Millipore). The concentrated media was then applied to the cancer cells after overnight starvation in 1% FBS. Cell lysis and blotting were then done. For the MDA-MB-175-VII cells, immunoprecipitation with anti-ErbB3 followed by blotting with anti-phosphotyrosine antibodies was performed. For the MDA-MB-453, MDA-MB-468 and MCF7 lines, immunoprecipitation with anti-MAPK followed by blotting with anti-phospho-MAPK was done. Results from these blots did not show any difference between the control and cells treated with the potential inhibitors.

In order to check that the inhibitors were being efficiently produced we wished to examine protein levels. The proteins are Myc-tagged but the tag is on the C-terminal part of the proteins, which is cleaved during secretion, and is likely degraded and therefore not useful for detection of secreted protein. Hence, an HA tag was put on the N-terminal portion via PCR. A small portion of the N-terminal region is also cleaved during the secretion process, so the HA tag was placed inside this cleavage point. Repeated attempts were made to detect the HA-tagged proteins. Conditioned media, concentrated conditioned media, and whole-cell lysates were assayed. Positive controls confirmed the transfections were successful and a non-secreted positive control for the HA tag demonstrated the antibodies were working.

Task 2. To generate and analyze the phenotypes of transgenic mice that express NRG-1: ΔEGF in heart and breast.

2 a. Creation of NRG-1: ΔEGF transgene for expression in early embryos

We proposed to test the ability of the mutant neuregulins to function as dominant negative proteins that mimic loss of function heart phenotypes seen in neuregulin knockout mice [3]. To do this we made a construct suitable for generating transgenic mice that express the gene in the heart under control of the α-myosin heavy chain promoter. We also made an additional construct with the EGF domain from Argos (NRG::Aos-EGF) because this factor appears to be a more potent inhibitor in flies (Fig 1). Furthermore Vinos and Freeman (2000)[2] showed that some mammalian cell types can indeed be inhibited by *Drosophila* Argos suggesting that an interaction between the Argos EGF domain and a vertebrate receptor is possible. Thus on balance it seemed prudent to make this additional transgene.

2 b. Generation and phenotypic analysis of NRG-1: ΔEGF transgenic mice

Neuregulin knockout mice die in mid embryogenesis of heart defects [3]. Thus we reasoned that the quickest way to test the efficacy of the inhibitors, which should act as dominant factors, would be to test whether they induce heart defects. To direct expression of the transgenes to the heart we used the α-myosin heavy chain promoter. In theory if the transgene were functioning as expected no transgenic mice should have been produced. Thus the hoped for result was negative; that no lines be produced. Nevertheless, it was essential that we conducted this experiment because the factors may not function in mice as they do in flies. If this were the case we would recover normal numbers of healthy transgenic mice. It turned out that the results were not 'cut and dried'- we got some mice but at lower than expected frequencies.

One founder mouse with the NRG:ΔEGF construct was created, after several injection attempts that produced a total of only nine mice. This low recovery was in keeping with our hypothesis that the transgene would act as a dominant negative inhibitor of neuregulin and be embryonic lethal. However, the Children's Hospital transgenic facility suffered technical problems at the time our mice were generated. (We therefore decided to use an alternative facility on campus for subsequent injections, see below.)

F1 and F2 litters have been produced from the NRG:ΔEGF founder and Southern blotting shows copy number estimates for the transgene are between 1 and 10. To determine whether this line produces viable progeny because the transgene is not efficiently expressed in the heart we performed reverse transcription of RNA from tissue samples followed by PCR to amplify transgene specific sequences (RT-PCR). Initially expression in the heart was not detected in all animals (as stated in the midterm report). However, assays with subsequent litters showed expression of the transgene in all transgenic individuals (multiple litters have been tested, two shown in Figs. 3 and 4).

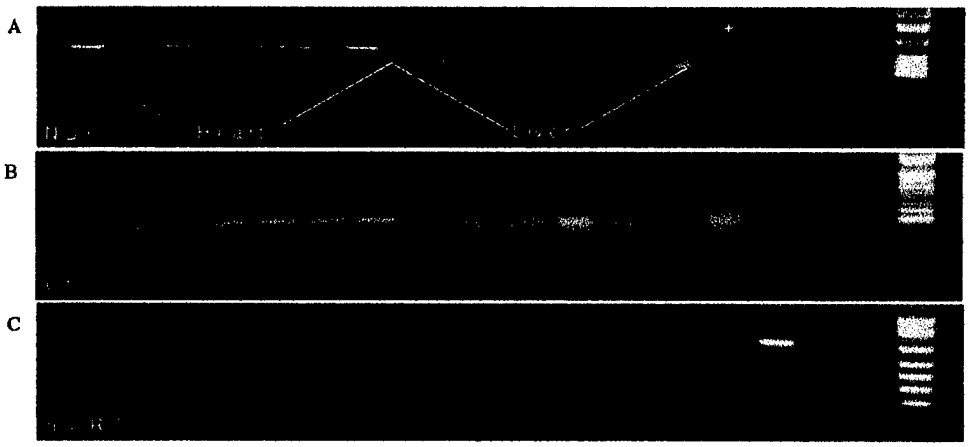

Figure 3. RT-PCR with an F1 NRG:ΔEGF litter. A. Transgene-specific primers. Only the transgenic mice (1,2,3,5,6,7) showed expression in the heart. B. Alpha-myosin heavy chain primers, positive control. C. No-RT control.

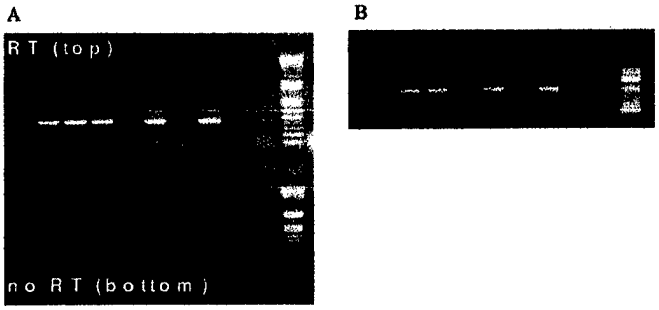

Figure 4. RT-PCR with an F2 NRG:ΔEGF litter. A. Transgene-specific primers, showing expression in hearts of the transgenic mice (no expression seen in the liver samples, data not shown). B. PCR screen of the F2 litter to compare with RT results, showing that only transgenic animals (1-3, 5 and 7) showed expression.

We are also testing NRG::Aos-EGF, which appears to be a stronger inhibitor in flies, and thus switched our efforts into analyzing transgenic mice with this construct. To test the function of NRG::Aos-EGF transgene we used the Keck transgenic mouse facility at Ohio State University. This transgenic mouse facility guarantees the production of at least 30 candidate mice of, which typically about half are transgenic. The NRG::Aos EGF construct yielded only 1 transgenic founder out of 22 mice and this mouse failed to transmit the transgene. This failure to generate a transgenic line is in keeping with our original hypothesis that the transgene is dominant lethal, but technical problems could also explain the result.

We thus tested directly for embryonic lethality caused by the NRG::Aos-EGF transgene in a transient transgenic experiment. Injections were performed and the embryos were harvested at day E11. A total of 23 embryos were tested and 7 of these were found to be transgenic. No gross heart defects in the embryos were observed by our colleague Dr. Michael Weinstein. Dr. Weinstein is an experienced mouse embryologist. Two transgenic embryos and two non-transgenic embryos were sectioned and their hearts examined in detail. No defects were detected (Figs. 5 and 6). In neuregulin knock out mice, trabeculation defects are seen by day E11 [3]. *In situ* hybridization showed that the transgene was expressed in the heart, albeit at lower levels than the alpha-myosin heavy chain control (Fig. 7). Despite transgene expression, trabeculation appeared normal. These results are consistent with the idea that the transgene is not acting as a neuregulin inhibitor and that the failure to recover stable lines was due to technical problems in generating the transgenic mice. Still, we cannot exclude the possibility that the transgene is causing a lethal phenotype (hence the failure to recover stable lines) and that a heart defect develops later than day E11. It would not be surprising to find that misexpression of an inhibitor fails to mimic the null phenotype exactly.

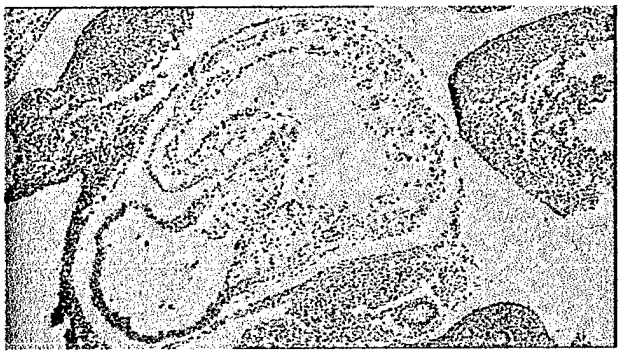

Figure 5. Cross-section of a wild-type day 11 embryo heart, showing normal trabeculae.

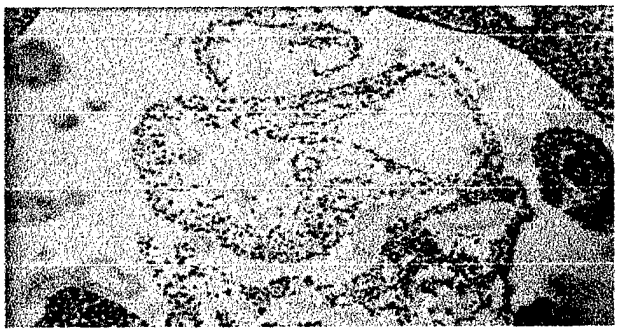

Figure 6. Cross-section of a NRG::Aos-EGF transgenic day E11 embryo. The embryo has normal trabeculation similar to its wild-type littermate (Figure 5).

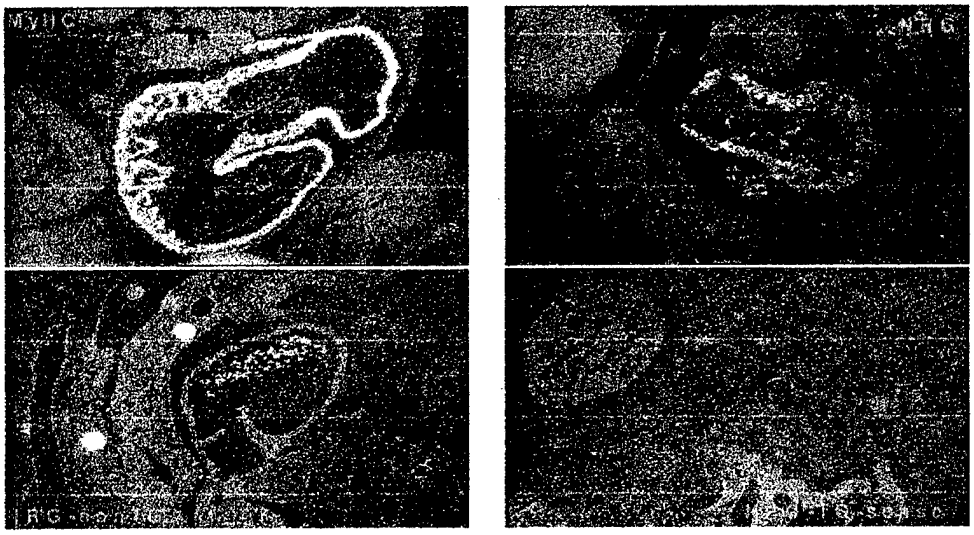

Figure 7. *In situ* hybridization in day E11 embryos,. Expression of NRG::Aos–EGF is seen in the heart (anti-sense probe, top right; sense probe, bottom right). Transgene expression was driven with the α-myosin heavy chain promoter and strong expression of α-myosin heavy chain is seen in the heart (top left). Non transgenic mice do not show expression demonstrating the probe is specific to the transgene (bottom left).

NRG::Aos EGF mice are viable

To resolve the issue of whether later defects could be caused by NRG::Aos-EGF expression we recieved a no cost extension to continue the work. During this period we had an additional injection to create NRG::Aos EGF transgenic mice conducted by Jon Neumann at the University of Cincinnati. This is an outstanding facility well known for the production of transgenic mice. Thirty-five mice were produced and 2 of these were found to be transgenic. A total of 5 litters were produced from the two founders. All litter sizes were normal and the mice were healthy. RT-PCR results of an F1 litter (n=10) showed that all 4 of the transgenic animals expressed the NRG::AOS construct in the heart but not the liver (Fig. 8).

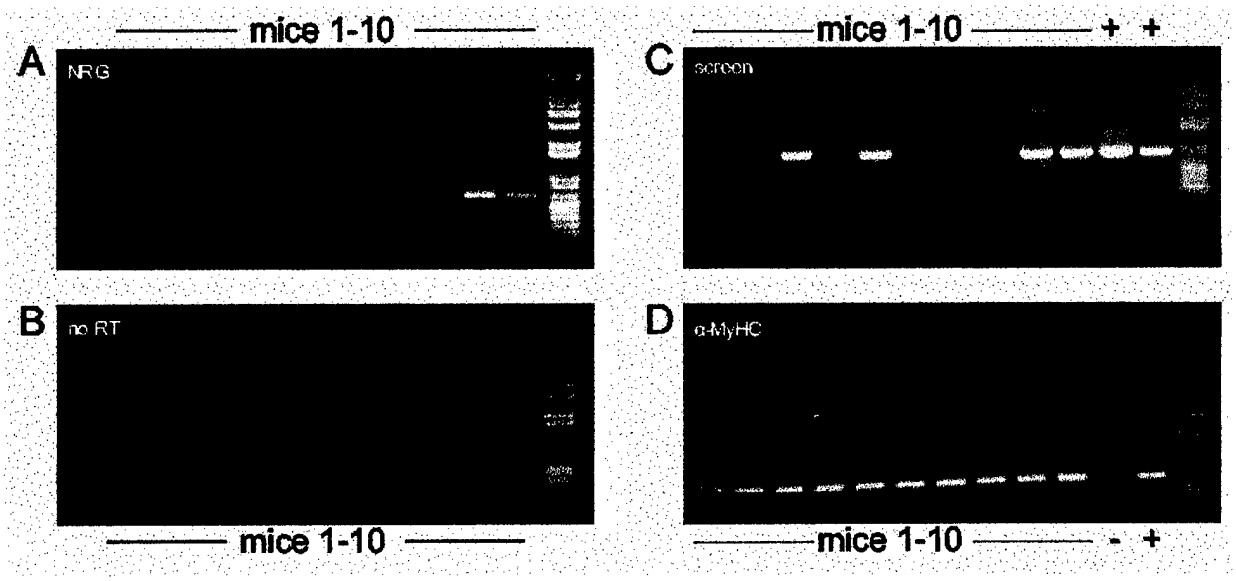

Figure 8. RT-PCR, NRG::AOS F1 litter. A. Expression in the hearts, seen in the transgenic mice (compare with C). B. No-RT control. C. PCR genotyping. D. RT-PCR, α-MyHC as positive control. For C and D, the "+" and "-" denote positive and negative controls, respectively.

Finding that the mice express and transmit the gene without detrimental effects we conclude that the inhibitor is unlikely to be useful in breast tissue. The remaining tasks (2 c&d and 3) described in our original plan were therefore not pursued as there is no sound rationale for initiating these studies at the present time.

Structure-function analysis of the Drosophila neuregulin

To gain more insights into the function of neuregulins we also used the no cost extension period to pursue additional studies in Drosophila. We have found key regions in the protein and have written a paper describing the work that is currently in review at Genetics (see appendix). The USAMRMC have been acknowledged for support of the work.

Key Research Accomplishments

- Demonstration that expression of a NRGΔEGF transgene in the heart does not cause lethality in mice
- Demonstration that transgenic mice expressing NRG::Aos EGF do not have heart defects on day E11
- Demonstration that expression of a NRG::Aos EGF transgene in the heart does not cause lethality in mice

Reportable Outcomes

- Department of Defense Era of Hope Meeting, Orlando, Florida, 2002: Transgenic Studies of a Modified Neuregulin (Jon Butchar, Michael Ostrowski, Michael Weinstein and Amanda Simcox) Poster
- Development of NRGΔEGF and NRG::Aos EGF transgenic lines that are apparently normal
- Manuscript in review (appendix)

Conclusions

Work with erbB signaling in *Drosophila* showed that a stimulatory factor, the fly neuregulin called Vein, could be converted into an inhibitor by either deleting the EGF domain or inserting the EFG domain from a natural inhibitor called Argos [1] (Fig. 1). This prompted the question as to whether such mutant factors would function similarly in vertebrates. To investigate this possibility we have generated transgenic mice with copies of altered neuregulin (NRG). Studies with transgenic mice carrying the deletion construct, NRGΔEGF, showed that expression of transgene in the heart did not cause a detectable phenotype. Work with *Drosophila* showed that the factor containing the EGF domain from Argos was a stronger inhibitor than the deletion form (Fig. 1). Therefore we also examined the activity of a NRG::Aos EGF transgene in mice. We studied this gene in transient transgenic experiments. Embryos injected with the NRG::Aos-EGF transgene were harvested and examined for heart defects at day E11. $NRG^{-/-}$ mice show trabeculation defects by this stage [3], but, no such defects were seen following expression of NRG-Aos EGF in the heart. However, misexpression of an inhibitor may not faithfully recapitulate the null phenotype and the embryos may show defects later in development. We tested this possibility by generating two mice with the NRG::Aos-EGF transgene. These were also normal. Therefore we conclude NRG::Aos EGF fails to inhibit NRG function in the heart. In sum, this work has allowed us to judge the efficacy of an inhibitory NRG developed in Drosophila. The results from the work suggest that the factors are not functioning in the mouse as they do in the fly.

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3. Meyer D, Birchmeier C. 1995. Multiple essential functions of neuregulin in development. *Nature* **378**: 386-90.

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APPENDIX

Regulation of the Drosophila EGF-ligand Vein is mediated by multiple domains

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ABSTRACT

Vein (Vn), a ligand for the Drosophila epidermal growth factor receptor (Egfr), has a complex structure including a PEST, Ig, and EGF domain. We analyzed the structure-function relationships of Vn by assaying deletion mutants. The results show each conserved domain influences Vn activity. A PEST deletion increases Vn potency and genetic evidence suggests Vn is regulated by proteasomal degradation. The Ig deletion causes toxic effects not seen following expression of native Vn, but the Ig domain is not required for Vn localization or for the activation of Egfr signaling in wing vein patterning. Remarkably, when the EGF domain is deleted, Vn functions as a dominant negative ligand, implying Vn normally physically interacts with another factor to promote its activity. We identified additional highly conserved sequences and found several regions that affect Vn potency and one which may mediate the effect of dominant negative Vn molecules. Together the results show that the activity of Vn is controlled both positively and negatively, demonstrating the existence of additional levels at which Egfr signaling can be regulated.

INTRODUCTION

Intercellular communication is fundamental to the development of multicellular organisms and facilitated by a number of signaling systems. The ErbB receptor family has four vertebrate members, the epidermal growth factor receptor Egfr/ErbB1, ErbB2/neu, ErbB3, and ErbB4, which play key roles in cell communication by acting as receptors for EGF-like signals including EGF, TGF-α, the neuregulins and others (FALLS 2003; HARRIS *et al.* 2003; OLAYIOYE *et al.* 2000). Drosophila has a single member of the ErbB family, Drosophila Egfr, and its activity is modulated by five ligands. The TGF-α-like molecules Gurken (Grk), Spitz (Spi), and Keren and the neuregulin-like molecule Vein (Vn) function as receptor activators (NEUMAN-SILBERBERG and SCHÜPBACH 1993; REICH and SHILO 2002; RUTLEDGE *et al.* 1992; SCHNEPP *et al.* 1996; URBAN *et al.* 2002). The fifth Egfr ligand, Argos (Aos), is a receptor antagonist (FREEMAN *et al.* 1992; SCHWEITZER *et al.* 1995a). ErbB receptors regulate many different cellular processes such as proliferation, cell survival, cell migration, and differentiation. Not surprisingly, aberrant activity of the receptors or their signaling components leads to a number of pathological outcomes (HOLBRO *et al.* 2003; OLAYIOYE *et al.* 2000).

Signaling through ErbB receptors is initiated when ligands bind to the extracellular domain, which relieves autoinhibition and exposes a dimerization loop within the receptor (FERGUSON *et al.* 2003; GARRETT *et al.* 2002; OGISO *et al.* 2002). Thus the activity of a ligand and the regulation of its production and presentation are key to signaling control as they precede all other events in the pathway.

The activity of a ligand is determined in part by the sequence of the EGF motif, which is required for receptor binding. For example, the vertebrate neuregulin-1 gene (*NRG*-1) encodes isoforms that differ in the EGF-like domain such that the β form is 10-100 times more potent

than the α form (FALLS 2003; LU *et al.* 1995). In Drosophila, the Spi EGF motif is a stronger activator of Egfr than the Vn EGF motif (SCHNEPP *et al.* 1998). EGF motifs are comprised of six conserved cysteine residues which form three disulfide bonds to generate a three-looped structure (the A, B and C loops), as well as a few other highly conserved residues. Whereas the overall sequences of the EGF motifs of Spi and Vn are about 40% conserved, the Aos EGF motif is significantly different. Notably, the Aos B-loop (the region between cysteines 3 and 4) is 20 amino acids long, compared to 10-12 amino acids in the activating ligands. Biochemical studies showed that Aos competes with agonists and prevents receptor dimerization (JIN *et al.* 2000). Although there is currently only one known homolog of Drosophila Aos, also in an insect, *Musca domestica* (HOWES *et al.* 1998), understanding how Aos functions is of considerable interest and may lead to the development of vertebrate Egfr inhibitors that could have therapeutic use in human disease.

HOWES *et al.* (1998) investigated structure-function relationships of the ligands Spi and Aos by creating a set of chimeras between the two proteins. They found that swapping the EGF domains of these proteins eliminated their function; neither the Spi EGF domain in Aos nor the Aos EGF domain in Spi had activating or inhibiting properties. In contrast, a chimeric molecule in which the Aos EGF domain was swapped with that of Vn (Vn::Aos-EGF) resulted in the conversion of the activator Vn into an inhibitor (SCHNEPP *et al.* 1998). The simplest interpretation of this result is that the Aos EGF motif is sufficient for receptor inhibition. However, here we report surprisingly that a Vn molecule completely lacking an EGF domain is also an inhibitor. Thus both molecules function as dominant negative ligands suggesting that the Aos EGF domain may not play a significant role in the inhibitory properties of Vn::Aos-EGF.

The creation of such dominant negative EGF-like ligands has implications for both normal regulation of ligand activity and the development of therapeutic inhibitors.

In addition to the intrinsic properties of a given EGF motif, ligand activity is regulated by transcriptional and post-translational mechanisms. Feedback loops involving transcriptional regulation of the ligand genes *vn* and *aos* have been discovered in Drosophila (GOLEMBO *et al.* 1996b; 1999; WASSERMAN and FREEMAN 1998; WESSELLS *et al.* 1999). These function to spatially refine signaling and ensure robustness (CASCI and FREEMAN 1999; SHILO 2003). Post-translational processing of ligands is also important in Drosophila Egfr signaling, where proteolytic cleavage activates Spi and the other TGF-α-like ligands. The trafficking and cleavage of the ligands is mediated by the membrane proteins Star and Rhomboid (Rho) (BANG and KINTNER 2000; GHIGLIONE *et al.* 2002; LEE *et al.* 2001; REICH and SHILO 2002; TSRUYA *et al.* 2002; URBAN *et al.* 2001; 2002). Star, is required for transporting the membrane-tethered ligands from the ER to the Golgi where they are cleaved by the intramembrane serine protease Rho and then secreted by the normal route.

Unlike the TGF-α agonists, Vn is made as a secreted molecule and is not dependent on proteolytic activation. *vn* is expressed in a spatially restricted pattern (SCHNEPP *et al.* 1996; SIMCOX *et al.* 1996), however, it is clear that transcription of *vn* per se does not always lead to an active ligand. Ectopic expression of *vn* in the early wing disc, where it acts as the key activator of Egfr signaling, does not mimic the transformations induced by ectopic expression of a consitutively active receptor (WANG *et al.* 2000; ZECCA and STRUHL 2002). This implies other factors are required to promote or inhibit Vn activity. Understanding the structure-function relationships of Vn will give insight into possible domains through which these factors may act.

Structurally, Vn resembles the vertebrate neuregulins because it possesses an Ig domain in addition to the EGF motif. The neuregulins exist in multiple different isoforms and those containing an Ig domain are essential for viability (KRAMER *et al.* 1996; MEYER and BIRCHMEIER 1995). Here we sought to understand the roles of the EGF, Ig, and other key domains in Vn. The results suggest Vn interacts with multiple factors that control its activity both positively and negatively, thus providing additional levels at which Egfr signaling can be regulated.

MATERIALS AND METHODS

Sequencing of *vein* EMS alleles: The *vein* alleles (allele name/synonym) sequenced were: *L6, WA178, ddd-2/RD310, ddd-3/RG436, ddd-7/UH5, ddd-10/VK97, ddd-11/VU288, ddd-12/VW100, ddd-13/WB240.* Mutant larvae were either homozygous for a given allele or transheterozygous with *Df(3L)vn-β3*. Genomic sequences were obtained by PCR amplification using Platinum Taq polymerase (Invitrogen). Exon 1 of *vn* was amplified as a 1.68 kb PCR product. Exons 2, 3, 4, and 5 comprising the remaining 523bp of the *vn* coding sequence were amplified as a contiguous 1.76 kb PCR product that included 1.24 kb of intronic sequence. Three clones for each allele as well as *vn* sequences from w^{1118} and *mwh red e* controls, were sequenced. Primers used are listed in supplemental material.

Cloning *D. virilis vn*: Reverse transcription was performed by standard procedures using a RETROscript™ (Ambion) kit and 2 μg total *D. virilis* RNA as a template and Oligo(dT) as the primer. Degenerate primers were used to amplify *vn* sequences and a FirstChoice™ RLM-RACE (Amicon) kit was used to complete the 3' end of the gene. Primers used are listed in supplemental material.

Generation of Vn transgenes: *Vn::Aos/Spi-EGF chimeras:* Recombinant PCR was employed using Vn::Aos-EGF or Vn::Spi-EGF in pBS (SCHNEPP *et al.* 1998) as templates to take advantage of the XmaI and SpeI sites flanking the EGF motif. The inside primers (1-8, supplemental material) corresponded to sequences within the EGF motif flanking the junctions between the A, B, or C loops and contained both Aos and Spi sequence; the outside primers corresponded to sequences in the Vn backbone or pBS T7.

Vn:ΔEGF: pBS-Vn1 was used as a template for two PCR reactions, one using primers pBS T3 and DEGF-R and the other using pBS T7 and DEGF-F. The products, corresponding to

residues 1-564 and 599-622, respectively, were cut with XmaI, ligated and cloned into the EcoRI site of pBS.

Vn:ΔIg: pBS-Vn1 was used as a template for PCR with primers pBS T7 and DIg-F. The PCR product, corresponding to residues 522-622, was cut with BglII/Not1 and used to replace the corresponding fragment in pBS-Vn1.

Vn:ΔMR$^{93\text{-}213}$: The orientation of the Vn1 cDNA in pBS was reversed and the resulting construct (named pBS-Vn1^{R}) was used as a template for PCR with primers T7 and MR3-R. The PCR product, corresponding to residues 1-92, was cut with EagI and used to replace the corresponding fragment in pBS-Vn1^{R}.

Vn:ΔMR$^{177\text{-}395}$: pBS-Vn1 was cut with SphI, purified to remove an internal SphI fragment corresponding to residues 177-395 and religated.

Vn:ΔMR$^{395\text{-}476}$: pBS-Vn1 was used as a template for PCR with the primers pBS T7 and MR2-F. The PCR product, corresponding to residues 477 to 621, was cut with SacII and used to replace the corresponding fragment in pBS-Vn1.

Vn:ΔPEST: pBS-Vn1 was used as a template for PCR using primers pBS T3 and DP-R. The product, corresponding to residues 1-41, was cut with AflII/XhoI and used to replace the corresponding fragment in pBS-Vn1.

Constructs were excised from pBS and inserted into the transformation vector pUAST. Transgenic stocks were generated by standard techniques and multiple transgenic lines for each construct were examined (see below). Primer sequences are listed in supplemental material.

Drosophila stocks and cultures: All crosses were performed at 25°C, unless otherwise noted. All Gal4 lines (*71B, 69B, bs-1348, en, Kr, ptc*) and the *DTS5* and *DTS7* proteasome subunit alleles were obtained from the Bloomington Stock Center. To account for differences in

expression due to position effects, we analyzed at least five independent transgenic lines for each construct, except for the Aos/Spi chimeras, when a minimum of 2 lines was examined (Table 1). There were only two cases (Vn:ΔIg and Vn:ΔMR^{93-213}) when an individual line exhibited a phenotype that was somewhat weaker than the others in that group. The stronger lines were used unless otherwise indicated.

Expression analysis: Embryos were prepared and processed for in situ hybridization using standard procedures (TAUTZ and PFEIFFLE 1989) and mounted in Aquapolymount (PolySciences, Inc.) for analysis by brightfield microscopy. Immunostaining of embryos was performed using standards procedures (PATEL 1994). A rat polyclonal to Vn (kindly provided by T. Volk (YARNITZKY *et al.* 1997)) was diluted 1:200, Cy3-conjugated goat-anti-rat secondary (Jackson) was diluted 1:500. Embryos were mounted in Vectashield (Vector Laboratories) and analyzed with a Biorad MRC 1024 confocal laser microscope.

Bromodeoxyuridine (BrdU) labeling: Larvae were dissected in Schneider cell medium and inverted anterior ends were incubated in Schneider cell medium containing 50 μg/mL BrdU (Roche Molecular Biochemicals) for 30 minutes to label proliferating cells. BrdU detection was as previously described (HARTENSTEIN and POSAKONY 1989) with mouse anti-BrdU (Becton-Dickinson) used 1:20 and a goat-anti-mouse HRP conjugated secondary (Jackson) used 1:300. Tissues were mounted in Aquapolymount for analysis by brightfield microscopy.

RESULTS

Temperature-sensitive mutations map to the Ig and EGF domain: Nine EMS-induced *vn* alleles were sequenced (Figure 1A). The six nonsense mutations map to Exon 1 and thus either produce truncated proteins or would be subject to nonsense-mediated mRNA decay. The *WA178* allele contains a change at the first position of the intron at the Exon 2/Intron 2 splice junction, a position that is normally invariant (MOUNT *et al.* 1992). If splicing at this site does not occur, the read-through product would terminate at a premature STOP codon in Intron 2. The two missense mutants, *ddd-13* and *ddd-11*, each contain a single amino acid change, in the Ig domain and the EGF domain, respectively. Both *ddd-13* and *ddd-11* are temperature-sensitive mutations, suggesting that these two regions are key determinants of Vn structure and function. This has been confirmed by examining deletion mutants as described below.

Comparison with *Anopheles gambiae* and other Drosophila species reveals additional highly conserved regions: In order to determine if there are conserved regions in addition to the Ig and EGF domains, we compared the Vn sequences from other Drosophilids and a mosquito. The genomes of *Anopheles gambiae* and *Drosophila pseudoobscura* were recently sequenced (HOLT *et al.* 2002) (Human Genome Sequencing Center, Baylor College of Medicine, http://hgsc.bcm.tmc.edu/projects/drosophila/). The *A. gambiae vn* cDNA sequence was further confirmed by RT-PCR (data not shown). We also cloned and sequenced the *D. virilis vn* gene using degenerate RT-PCR and RACE (see Materials and Methods).

The structure of the *vn* gene is conserved between the Drosophilids and *A. gambiae*, with an exon coding for most of the protein followed by one large intron and several small exons that encode the Ig and EGF domains (data not shown). Interestingly, the EGF domain in each *vn* gene is divided by an intron located between the fourth and fifth cysteines. STEIN and STAROS

(2000) report that genes for vertebrate ErbB ligands contain a splice site in the same position. The placement of this intron appears to be unique to ErbB ligands and is not generally seen in other EGF domain containing proteins (STEIN and STAROS 2000), suggesting that the insect and vertebrate genes share a common ancestry.

The overall identity of the *D. melanogaster* Vn protein is 70% with *D. pseudoobscura*, 58% with *D. virilis*, and 26% with *A. gambiae*. The sequences of the Ig (Figure 1C) and EGF (Figure 1D) domains from these species are strongly conserved. The proline triplet at the beginning of the Ig domain that is affected in the conditional *ddd-13* mutant is conserved between all four proteins. There is also a high degree of similarity in the region just N-terminal to the Ig domain, which we term the mosquito conserved region (MCR, Figure 1B). The N-terminal portion of the gene has lower conservation (data not shown). As expected for a region with strong evolutionary conservation, deletions removing parts of the MCR impair the function of Vn (see below).

Vn::Aos/Spi-EGF chimeras function as inhibitors: A chimeric molecule comprised of the Aos EGF domain inserted into the Vn backbone (Vn::Aos-EGF) inhibited Egfr signaling (SCHNEPP *et al.*, 1998; Figure 2B). In an attempt to define the region within the Aos EGF motif conferring this property, we created a set of chimeric Vn molecules with EGF motifs corresponding to all possible combinations of the A, B, and C-loops from the Spi and Aos EGF motifs (Vn::Aos/Spi-EGF chimeras, Figure 2C). We chose to test chimeras between the Spi and Aos EGF motifs rather than the Vn and Aos EGF motifs because it has been shown that Spi is a stronger activator than Vn (GOLEMBO *et al.* 1999; SCHNEPP *et al.* 1998), and thus the difference in activity of the chimeric EGF motifs would be more apparent.

We tested the activity of each chimera using the Gal4-UAS system (BRAND and PERRIMON 1993). *UAS-transgenes* encoding the chimeras were misexpressed in the wing and their ability to produce vein loss, characteristic of Egfr inhibitors, or ectopic veins, characteristic of Egfr activators, was assessed. Surprisingly, every one of the chimeras functioned as an inhibitor (Table 1). Each EGF motif chimera had approximately the same activity (one shown in Figure 2D), which was also similar to that of Vn::Aos-EGF (Figure 2B), except A3S4A (Aos A and C loops with the Spi B loop, Table 1) that had weaker activity. We suspect this chimera acts as a weak inhibitor due to a non-specific defect rather than an effect of the Spi B loop because the two other chimeras that include this region (A3S and S4A) are potent inhibitors.

Vn:ΔEGF functions as an Egfr inhibitor: Finding that all Vn::Aos/Spi-EGF chimeras tested functioned as inhibitors suggested that the sequence of the Aos EGF motif was not critically important for mediating the inhibitory effect of Vn::Aos-EGF and the mechanism of inhibition was distinct from that of native Aos. One explanation could be that all chimeras possess non-functional EGF domains which cannot bind Egfr but interfere with signaling in a dominant negative fashion by affecting a pathway component other than the receptor. Therefore we tested whether an EGF domain was required for inhibition by creating a form of Vn lacking the EGF domain (Vn:ΔEGF). If the inhibition was occurring through a dominant negative mechanism not involving receptor binding, then Vn:ΔEGF would also be expected to function as an inhibitor. Indeed this was found to be the case.

Misexpression of Vn:ΔEGF with *69B-GAL4* produced a vein loss phenotype in the wing similar, although somewhat milder, to that produced by misexpression of Vn::Aos-EGF and the Vn::Aos/Spi-EGF inhibitors (Figure 3A). These phenotypes also closely resemble that of a hypomorphic mutation of *vn* (PURO 1982 and Figure 3B), further suggesting that the transgenes

are functioning as inhibitors through a dominant negative mechanism, possibly by interfering with the activity of endogenous Vn. In this model, Vn activity would be compromised because the inhibitors compete with Vn for a factor required to promote ligand-receptor interaction.

One prediction of this model is that in addition to being able to prevent endogenous Vn from activating Egfr, Vn:ΔEGF and Vn::Aos-EGF (hereafter referred to collectively as DN-Vn ligands) would also be able to inhibit misexpressed, native Vn. To test this, wild type Vn and DN-Vn transgenes were co-expressed using *69B-GAL4* and the resulting wing phenotypes were analyzed. We found that expression of Vn alone caused a moderate extra vein phenotype (Figure 3C) and this phenotype was suppressed by co-expression of DN-Vn (Figure 3D).

Vn:ΔIg has normal activity in wing vein patterning but is toxic in early development: Misexpression of Vn:ΔIg in pupal interveins (with *1348-GAL4*) produced a similar extra-vein phenotype to that caused by misexpression of native Vn (Figure 4A and B). This indicates that the Ig domain is not required for Vn-mediated receptor activation and that without an Ig domain, Vn has a similar ability to activate the receptor. In the neuregulins, the Ig domain is required for anchoring to the ECM (LI and LOEB 2001; LOEB and FISCHBACH 1995; LOEB *et al.* 1999), but this does not appear to be the case for Vn as Vn:ΔIg is concentrated at the surface of cells in which it is expressed (Figure 4C).

However, the Ig domain clearly has a function because the effect of Vn:ΔIg differed from wild-type Vn when misexpressed earlier in development. Misexpression of Vn:ΔIg resulted in complete lethality with *71B-GAL4*, whereas misexpression of wild-type Vn only slightly affected viability. Expression of wild-type Vn with *69B-GAL4* Vn induced partial lethality but this occurred at the pupal stage, whereas misexpression of Vn:ΔIg was embryonic lethal (data not

shown). We found one transgenic line of Vn:ΔIg that produced adults with *69B-GAL4*, presumably because the insertion site of the transgene supports only relatively low expression levels. These exhibited a moderate extra-vein phenotype (Figure 4F) similar to that caused by ectopic expression of native Vn, but this Vn:ΔIg line also consistently produced serrated wing margins, which were only rarely seen following Vn misexpression, demonstrating an abnormal function for the Ig deletion mutant.

Finally, misexpression of Vn:ΔIg with *T80-GAL4* resulted in bloated larvae (Figure 4D). The brains and imaginal discs in these larvae were smaller than those of wild type and showed reduced levels of BrdU incorporation (Figure 4E), indicating a defect in cell proliferation in these tissues. Misexpression of native Vn caused mild larval bloating but did not result in either of these disc or brain phenotypes. Together these data show that the Ig domain is not required for Vn to function as an activator, but nevertheless indicate a role for the Ig domain in modulating Vn activity because expression of Vn:ΔIg is toxic early in development.

The dominant negative effect of DN-Vn is not mediated by the Ig domain: The Ig domain is a known protein-protein interaction domain and therefore a candidate region for mediating the effect of the DN-Vn ligands such that the phenotypes caused by these ligands may result from sequestering a positive factor normally bound by the Ig domain of Vn. To test this, we generated flies expressing DN-Vn constructs in which the Ig domain was deleted (Vn::Aos-EGF-ΔIg and Vn:ΔEGF-ΔIg). These proteins were robustly expressed and localized in the ECM (not shown) but had no detectable effect on Egfr signaling. This suggests either that the Ig domain is required for the function of the DN-Vn inhibitors or that the proteins are inactive due to a non-specific effect. To distinguish between these possibilities, we tested for genetic interactions between DN-Vn and Vn:ΔIg. If the DN-Vn ligands were functioning through a

mechanism involving the Ig domain, then DN-Vn would not be expected to alter the phenotype of Vn:ΔIg, as this molecule would necessarily function independently of the Ig domain.

We analyzed the phenotypes resulting from co-expression of Vn:ΔIg and DN-Vn (with *69B-GAL4*). We found that the wing phenotypes caused by expression of the weaker Vn:ΔIg line were rescued by co-expression of DN-Vn (Figure 4G). Furthermore, while expression of most Vn:ΔIg lines alone resulted in embryonic lethality (see above), we found that co-expression of DN-Vn rescued this lethality and produced viable adult progeny that exhibited a mild extra vein phenotype (not shown). Thus DN-Vn ligands are effective suppressors of Vn molecules lacking an Ig domain, suggesting that some other region mediates the dominant negative effect. We suggest this region is part of a highly conserved sequence (MCR) in the middle portion of Vn (see below).

Vn:ΔPEST is a stronger agonist than native Vn: PEST domains serve as signals for proteolytic degradation of proteins (RECHSTEINER and ROGERS 1996). Thus, if the PEST domain of Vn is functional, its removal would be expected to generate a more stable protein. Misexpression of Vn:ΔPEST with *69B-GAL4* and *71B-GAL4* caused lethality whereas misexpression with *1348-GAL4* resulted in a strong extra-vein phenotype (Figure 5B). This phenotype was more severe than that resulting from misexpression of wild-type Vn (Figure 5A), indicating that Vn:ΔPEST has enhanced signaling capacity, possibly through an increase in protein stability. Misexpression of Vn:ΔPEST resulted in a high level of Vn expression in embryos and the protein appeared to be more widely distributed and not limited primarily to the surface of cells (Figure 5C). This distribution could be a result of Vn persisting in the cells.

PEST domains are involved in targeting proteins to the 26S proteasome. In Drosophila, two mutants, *DTS5* and *DTS7,* affect the β6 and β2 proteasome subunit genes, respectively (MYKLES 1999). *DTS5* and *DTS7* heterozygous flies develop normally at the permissive temperature (25°C), but die in the pupal stage when grown at the restrictive temperature (29°C). Shifting to the restrictive temperature for 48 hours during the third larval instar allows the flies to survive to adulthood and these exhibit a mild extra vein phenotype (Figure 5 E, both alleles have vein spurs around L5, not shown). The *UAS-Vn*$^{1.1}$ line also has a mild constitutive extra vein phenotype caused by leaky expression of the transgene (Figure 5F). In combination with the *DTS5* and *DTS7* mutations, and following a shift to the restrictive temperature, this extra vein phenotype is dramatically enhanced (Figure 5G and H) suggesting that impairing proteasome function enhances Vn activity, possibly by reducing Vn degradation.

Amino acids 177-476 of Vn are required for full activity whereas amino acids 93-177 negatively regulate Vn activity: The Vn middle region (amino acids 96 to 476) lacks sequences with homology to known functional domains, although stretches of the region are highly conserved with the mosquito (Figure 1B). Deletion mutants spanning the middle region show that this region is indeed required for Vn function and that some parts promote activity while another negatively regulates Vn activity.

Vn:ΔMR$^{93\text{-}213}$, which removes amino acids 93-213, functioned as a super-strong activator. Misexpression of Vn:ΔMR$^{93\text{-}213}$with *69B-GAL4* and *71B-GAL4* caused embryonic and early pupal lethality, respectively. One line of Vn:ΔMR$^{93\text{-}213}$ appeared to be weaker than the others tested (presumably due to a position effect of the transgene insertion site). In this line, expression with *71B-GAL4* primarily caused lethality in late pupal/pharate adult stage but a few

escapers survived that exhibited a strong extra vein phenotype (Figure 6C). With expression induced by *1348-GAL4*, most of the Vn:ΔMR$^{93\text{-}213}$ lines produced a strong extra vein phenotype and wing blisters (Figure 6B). These phenotypes are much stronger than those seen following misexpression of native Vn (Figures 4A and 6A). In contrast, the mutants Vn:ΔMR$^{177\text{-}395}$ and Vn:ΔMR$^{395\text{-}476}$, which remove amino acid residues 177-395 and 395-476, respectively, both functioned as weak activators when compared to native Vn, producing mild extra-vein phenotypes with a strong driver (*69B-GAL4*) (Figure 6E and G). Together these results suggest amino acid residues 93-213 of the 'middle region' negatively regulate Vn activity whereas the remainder of the middle region is required for full Vn activity.

Amino acids 213-395 may mediate the dominant negative effect of DN-Vn ligands: The DN-Vn ligands are presumed to function by competing with Vn for binding to a factor that promotes Vn/Egfr interaction. The region responsible for binding is therefore expected to have a positive effect on Vn activity. Two middle region deletion mutants, Vn:ΔMR$^{177\text{-}395}$ and Vn:ΔMR$^{395\text{-}476}$, reduced the activity of Vn and are thus candidates for this region. To test this possibility, we determined whether co-expression of DN-Vn could suppress the extra vein phenotype induced by expression of these deletion mutants (we also tested Vn:ΔMR$^{93\text{-}213}$, although this was considered a less likely candidate as this deletion enhanced Vn activity). We analyzed the wing vein phenotypes resulting from co-expression of DN-Vn with each middle region deletion.

69B-GAL4; Vn:ΔMR$^{93\text{-}213}$ flies died as embryos and we observed no rescue of this lethal phase by co-expression of DN-Vn (not shown). However, when we co-expressed DN-Vn and the Vn:ΔMR$^{93\text{-}213}$ transgene that had the weakest effect with *71B-GAL4*, we found that more flies

survived to adulthood (compared to Vn:ΔMR$^{93-213}$ expression alone) and the extra vein phenotype of these flies was reduced (Figure 6C, D).

Co-expression of DN-Vn with *69B-GAL4* was also able to suppress the extra vein phenotypes resulting from overexpression of Vn:ΔMR$^{395-476}$ (Figure 6H). Expression of Vn:ΔMR$^{395-476}$ alone resulted in deltas at the distal tips of L3 and L4 as well as a thickening of the distal portion of L2. Co-expression of DN-Vn eliminated these extra veins.

In contrast, there was no suppression of the extra vein phenotype resulting from co-expression of Vn:ΔMR$^{177-395}$ and DN-Vn (Figure 6F). The deltas and thickenings observed at the distal portions of the lateral veins caused by misexpresson of Vn:ΔMR$^{177-395}$ were unaffected by co-expression of DN-Vn. This result implicates the sequences between residues 213 and 395 in mediating the effect of the DN-Vn ligands and as the region in native that Vn that is required for binding a factor that promotes Vn/Egfr interaction. This falls within a region we found to be highly conserved with the mosquito (MCR, Figure 1B).

Deletion mutants with enhanced activity in the wing do not influence embryonic cell fate: We found that each of the deletion mutants could at least partially rescue the wing disc phenotype of *vn* mutants (Supplemental Figure 1). However, the observation that several of the Vn deletion mutants tested (Vn:ΔIg, Vn:ΔPEST and Vn:ΔMR$^{93-213}$) differed from wild-type Vn in that they caused embryonic lethality suggested the possibility that these mutations were transforming Vn into a stronger agonist, more similar to Spi. Spi is more potent than Vn when misexpressed in embryos and results in an expansion of ventral cell fates, which can be monitored by examining the expression of *orthodenticle (otd)* (Figure 7A-C) (GOLEMBO *et al.* 1996a; 1999; SCHNEPP *et al.* 1998; SCHWEITZER *et al.* 1995b; WIESCHAUS *et al.* 1992).

Misexpression of Vn:ΔIg, Vn:ΔPEST or Vn:ΔMR$^{93\text{-}213}$ with *Kr-GAL4*, caused no expansion of *otd* expression (Figure 7D-F). This indicates that although two of these mutant forms, Vn:ΔPEST and Vn:ΔMR$^{93\text{-}213}$, have enhanced capacity to induce ectopic veins, and all three cause lethality when expressed in the embryo, they do not resemble Spi in their ability to induce ectopic ventral cell fates.

DISCUSSION

Mechanisms that govern production and presentation of an active ligand form the most fundamental levels of signaling control, presaging all other events in the pathway. Ligand activity can be controlled by both transcriptional and/or post-translational regulation. Transcriptional regulation is important for *vn,* which unlike the other zygotically active ligands *spi* and *keren*, is expressed in a highly localized and dynamic pattern (GOLEMBO *et al.* 1999; REICH and SHILO 2002; RUTLEDGE *et al.* 1992; SCHNEPP *et al.* 1996; SIMCOX *et al.* 1996; WESSELLS *et al.* 1999; YARNITZKY *et al.* 1997). Vn is made as a soluble protein and thus does not require processing like the membrane spanning TGF-α ligands. However, its sequence predicts that Vn is a complex molecule and here we have shown that the activity of Vn is indeed regulated through multiple functional domains that mediate both negative and positive effects on Vn activity. The results reveal additional levels of complexity through which Egfr signaling is controlled and we discuss the potential conservation of these mechanisms.

Deletion of the Vn EGF domain creates an inhibitor: Remarkably when the EGF domain is deleted, Vn becomes an inhibitor. The activity of this mutant molecule is similar to a chimeric ligand, Vn::Aos-EGF (SCHNEPP *et al.* 1998) that includes the EGF domain from Aos, the natural Egfr antagonist. We had previously ascribed the inhibitory function of Vn::Aos-EGF to possession of the Aos sequence but in light of the findings described here, it is likely both Vn::Aos-EGF and Vn:ΔEGF function by a dominant negative mechanism. This also allows us to reconcile the difference in the activities of Vn::Aos-EGF and Spi::Aos-EGF chimeras (HOWES *et al.* 1998; SCHNEPP *et al.* 1998); both share in common the Aos EGF domain, but only the

Vn::Aos-EGF chimera is able to function as an inhibitor of Egfr signaling through a dominant negative mechanism involving a critical domain found only in the Vn 'backbone'.

There are several possible models that could explain how these DN-Vn mutants are able to inhibit Egfr signaling. In the simplest model, Vn/Egfr signaling could involve dimerization of Vn. A dimer formed between Vn and DN-Vn would likely be inactive. Expression of DN-Vn would thus reduce the number of active Vn-Vn dimers and result in inhibition of Vn/Egfr signaling. However, the recent structure of Egfr in complex with its ligands excludes the possibility that a Vn dimer is part of the receptor-ligand complex because the two ligands are expected to be about 70-80Å apart on opposite sides of the complex (GARRETT *et al.* 2002; OGISO *et al.* 2002). However, this does not rule out the possibility Vn-Vn interactions have a role in subsequent multimerization of receptor dimers to form, for example, tetramers. In an alternative model, Vn/Egfr activation could depend on an interaction between Vn and another factor. In this case, overexpression of DN-Vn would compete for binding with this factor and abrogate Vn-mediated receptor activation.

Both models predict there must be a region in Vn that mediates the effect of the inhibitors by competing for binding to this factor. The normal role for this region is therefore to potentiate Vn function and hence deletion of the region should lower Vn activity. Furthermore, the model predicts that a molecule lacking the key region would not be influenced by the DN-Vn ligands. In our analysis of Vn deletion mutants we found two adjacent regions ($MR^{177-395}$ and $MR^{395-476}$) that reduced Vn function in an ectopic expression assay. While both deletions remove blocks of conserved sequences (MCR), only $Vn{:}\Delta MR^{395-476}$ was able to be suppressed by DN-Vn. This suggests that residues 213-395 are important for mediating the dominant negative

effect. It will be important to map the required region in more detail to determine if the MCR performs this role, but our data indicate that the C-terminal portion of the MCR (which is relatively less conserved) is not required.

One question that arises from this work is whether an inhibitory vertebrate ligand can be created. van de Poll et al. (1997) and Lohmeyer et al. (1997) generated EGF molecules with extended B loops in attempts to mimic Argos function. None of these factors had inhibitory properties. While we believe the inhibitory nature of Vn::Aos-EGF is primarily mediated through a dominant negative mechanism independent of the Aos sequence, we did note that the Vn::Aos-EGF chimera was a more potent inhibitor than Vn:ΔEGF because stronger induction of the transgene (elevating the rearing temperature to increase the activity of Gal4 and hence *UAS-transgene* expression) was required to produce an equivalent phenotype. This suggests that some intrinsic property of the Aos EGF domain is also having an effect. But basing the design of an inhibitor on the Aos EGF region is unlikely to be a successful approach given the results with vertebrate ligands and the lack of activity of Spi::Aos-EGF. Instead it may be efficacious to investigate vertebrate ligands that rely on binding with other factors to potentiate interaction with the receptor.

Vn activity may be regulated by protein degradation: Attenuation of signaling can be dependent on ligand destruction and structure-function analysis suggests Vn may be regulated by degradation. Deletion of two regions in the N-terminal part of Vn produced mutant proteins with increased ability to activate Egfr as judged by their ability to produce ectopic veins. One of these regions (amino acids 58-96) is strongly predicted to contain a PEST sequence (Figure 1)

by the PestFind algorithm (RECHSTEINER and ROGERS 1996 http://www.at.embnet.org/embnet/tools/bio/PESTFIND).

Our observation that the removal of the PEST domain of Vn results in a more potent activator suggests that Vn is subject to regulation by protein degradation. This would be a novel mechanism for regulation of an EGF ligand. In support of this idea we found a genetic interaction between a *vn* transgene and mutants for proteasome subunits. Analyzing this connection further will be important not only for understanding Vn regulation, but may have broader implications as PEST domains have been reported in two other EGF ligands, Gurken and Lin-3 (HILL and STERNBERG 1992; NEUMAN-SILBERBERG and SCHÜPBACH 1993), and can also be detected in the neuregulins (S-H Wang and A. Simcox unpublished). Therefore, any such degradation mechanism may be conserved and involve multiple ligands.

Different roles for the Ig domain in Vn and the vertebrate neuregulins: Of the four vertebrate neuregulin (*NRG*) genes, both *NRG*-1 and *NRG*-2 are alternatively spliced to produce isoforms that possess an Ig domain (FALLS 2003). The Ig domain in *NRG*-1 binds to heparin sulfate proteoglycans. This maintains a high local concentration of ligand that results in enhanced receptor activation and extends the duration of the response (LI and LOEB 2001). Although Vn resembles the Ig-containing *NRG* isoforms, we show here that the Ig domain in Vn is unlikely to have a similar role. Deletion of the Ig domain did not diminish the activity of Vn or prevent its association with the ECM. Instead the Vn:ΔIg mutant appeared to have additional properties and caused a number of detrimental effects when ectopically expressed that were not observed with native Vn. The evolutionary relationship of the vertebrate and invertebrate ligands is not clear. Certain residues in the EGF domain are characteristic of the neuregulins

(BUONANNO and FISCHBACH 2001), but these are not conserved in Vn suggesting that it may be no more related to the neuregulins than any other Drosophila EGF ligand. Furthermore, the Ig domains of Vn and the neuregulins appear to have at least some distinct functions.

Role of ligand regulation in cell signaling control: The analysis of the Drosophila TGF-α genes has highlighted the importance that processing of the membrane bound ligand precursors plays in signaling regulation. Here we show there is also a remarkable potential to regulate activity of the only secreted agonist, Vn. We found Vn activity was altered by deleting each of three known conserved domains (PEST, Ig and EGF) and also identified a novel domain that is required for activity. In subsequent analysis it will be important to define the mechanisms that govern these activities and to determine which if any are conserved in other animals.

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FIGURE LEGENDS

FIGURE 1. The Vn protein. (A) Structure of the Vn protein showing conserved domains and mutations associated with various alleles. (B-D) Alignment of Vn protein showing conserved regions from three Drosophilids and the mosquito (*Anopheles gambiae*): (B) novel conserved region (MCR, mosquito-conserved region); (C) Ig domain; (D) EGF domain. Similar (light shading) and identical (dark shading) amino acids are indicated.

FIGURE 2. Vn molecules with chimeric EGF domains function as inhibitors. (A) A wild type wing showing the normal pattern of wing veins. (B) Expression of *UAS-vn::Aos-EGF* with *69B-GAL4* inhibits Egfr activity and causes vein loss. (C) Cartoon showing chimeric EGF domains composed of all combinations of the A, B and C loops of Aos (black) and Spi (white). Each chimeric EGF domain was inserted in place of the Vn EGF domain and the resulting constructs were tested for effects on Egfr activity (Table 1). (D) Expression of *UAS-vn::Aos/Spi-EGF*^{S3A4S} with *69B-GAL4* causes vein loss (29°C, two copies of the transgene).

FIGURE 3. Vn:ΔEGF functions as a dominant negative inhibitor of Vn/Egfr signaling. (A) Misexpression of *UAS-vn:ΔEGF* with *69B-GAL4* results in partial loss of vein L4 (29°C), a phenotype that resembles the hypomorphic *vn*^{wvn} mutation (B). (C) Misexpression of *UAS-vn* in the wing results in an extra vein phenotype. (D) Co-expression of *UAS-Vn:ΔEGF* suppresses the extra vein phenotype.

FIGURE 4. Phenotypic effects of expression of Vn:ΔIg. (A-B) Similar extra vein phenotypes result from expression of *UAS-vn* (A) or *UAS-vn:ΔIg* (B) in pupal intervein regions with *1348-GAL4*. (C) The Ig domain is not required for the localization of the Vn protein. To allow for the identification of cells expressing the transgenes, *UAS-vn:ΔIg* and *UAS-GFP* were co-expressed in embryos using *en-GAL4*. Staining with a Vn antibody shows that the Vn:ΔIg protein remains closely associated with the cells in which GFP is expressed. Anterior is to the left. (D) Expression of *UAS-vn:ΔIg* with *T80-GAL4* causes bloated larvae (bottom), whereas expression of Vn causes only a slight effect (middle) compared to wild type (top). (E) Vn:ΔIg causes a reduction in cell proliferation in imaginal tissues. (Top) Pattern of BrdU incorporation in wild type brain (left) and wing, haltere, and third leg discs (right). Expression of *UAS-vn:ΔIg* causes a reduction on BrdU incorporation (bottom), whereas expression of *UAS-vn* does not (middle). Crosses in (D) and (E) were performed at 17°C. (F-G) The effect of DN-Vn is not mediated through the Ig domain. Expression of *UAS-vn:ΔIg* in the wing (using a weakly expressed transgene because most are lethal, see text) causes severe notching of the wing margin (compare with Figure 3C) and an extra vein phenotype (F). These phenotypes are rescued by co-expression of *UAS-vn:ΔEGF* (G).

FIGURE 5. Function of the PEST domain. (A-C) Deletion of the PEST domain enhances Vn activity. Expression of *UAS-vn:ΔPEST* (B) produces a stronger extra vein phenotype than *UAS-vn* (A) and results in increased intracellular Vn accumulation in the embryo (C, compare to Figure 4C). (D-H) The proteasome may play a role in Vn/Egfr signaling. Temperature-sensitive mutations in proteasome subunits β6 (D) and β2 (E) cause mild extra-vein phenotypes. The β6

(G) and β2 (H) mutations enhance the extra vein phenotype of a leaky *UAS-vn* line (F). Animals in D-H were shifted to 29°C for 48 hours during third larval instar.

FIGURE 6. The central region of the Vn protein contains sequences that both positively and negatively regulate Vn activity. (A-C) Amino acids 93-213 negatively regulate Vn activity. Compared to expression of *UAS-vn* with *71B-GAL4*, which causes only a slight extra vein phenotype (A), expression of the weakest *UAS-vn:ΔMR*$^{93\text{-}213}$ (stronger lines were lethal, see text) results in a smaller wing with a strong extra vein phenotype and notching (C). Expression of *UAS-vn:ΔMR*$^{93\text{-}213}$ in pupal interveins causes a strong extra vein phenotype and blistering (B, compare to Figure 4A). (D) Amino acids 93-213 do not mediate the dominant negative effect of DN-Vn. Co-expression of *UAS-vn:ΔMR*$^{93\text{-}213}$ and *UAS-vn:ΔEGF* partially rescues the extra vein phenotype of *UAS-vn:ΔMR*$^{93\text{-}213}$ expression. (E and G) Amino acids 177-476 are required for full Vn activity. Compared to expression of *UAS-vn* (Figure 3C), *UAS-vn:ΔMR*$^{177\text{-}395}$ (E) or *UAS-vn:ΔMR*$^{395\text{-}476}$ (G) with *69B-GAL4* cause very weak extra vein phenotypes (arrows). (F and H) Amino acids 213-395 may mediate the dominant negative effect of the DN-Vn ligands. Co-expression of *UAS-vn:ΔEGF* rescues the extra vein phenotype of *UAS-vn:ΔMR*$^{395\text{-}476}$ (H), but not *UAS-vn:ΔMR*$^{177\text{-}395}$ (F, arrows), suggesting the latter region, which includes part of the MCR, is important for mediating the effect of DN-Vn.

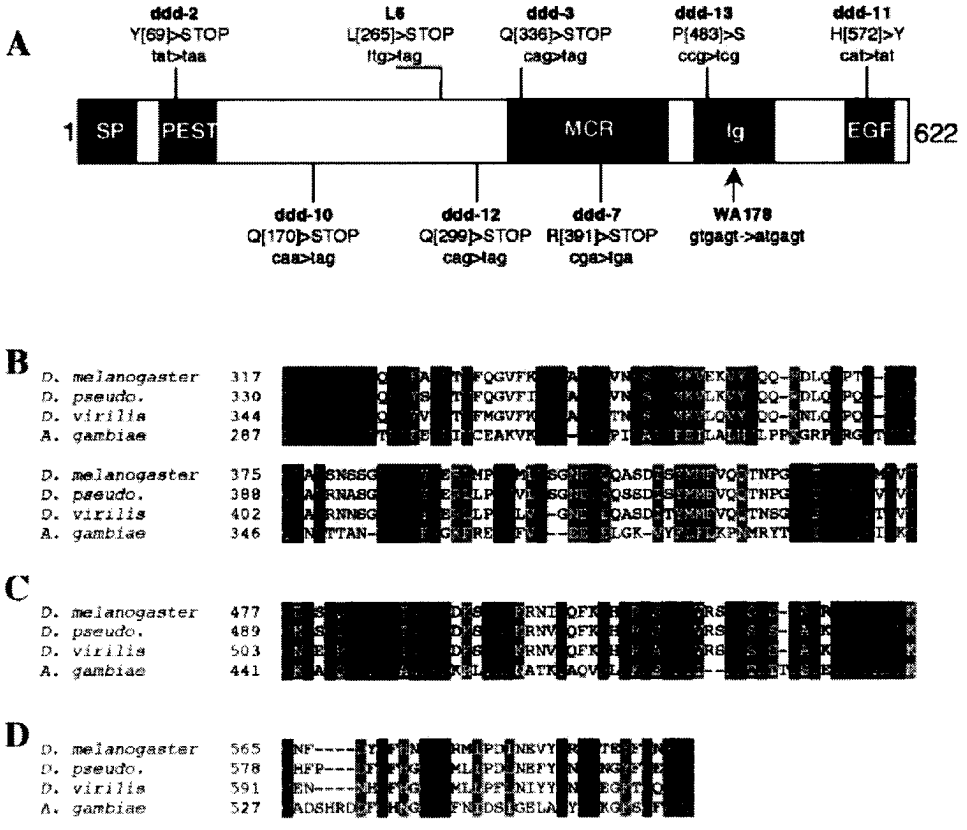
FIGURE 7. Ectopic expression of native Vn or the deletion mutants does not affect ventral cell fate determination. (A-F) Expression of *UAS-vn* (C), *UAS-vn:ΔIg* (D), *UAS-vn:ΔMR*$^{93\text{-}213}$ (E), or *UAS-vn:ΔPEST* (F) with *Kr-GAL4* does not alter the pattern of *otd* expression compared to wild

type (A), indicating that these factors are not like *UAS-sSpi* (B) in their ability to affect ventral cell fate determination.

Table 1. Vn::Aos/Spi-EGF chimeras function as inhibitors of Egfr signaling

UAS-transgene[a]	Number lines tested (60-130 wings scored/line)	Frequency of missing anterior crossvein (%) (range)[b]
lac-Z (control)	1	0
Vn::S3A	2	90 (85-94)
Vn::A3S4A	3	5 (0-9)
Vn::A4S	4	85 (79-98)
Vn::A3S	2	72 (62-82)
Vn::S3A4S	5	81 (62-95)
Vn::S4A	4	83 (74-92)
Vn::Aos-EGF	3	92 (87-99)
Vn::Spi-EGF	3	lethal[c]

[a] *ptc-GAL4* flies were crossed to each of the transgenic lines. *ptc-GAL4/UAS-x* female flies were examined for the presence of the anterior crossvein. The chimeric EGF domains are shown in Figure 2. [b] The anterior crossvein is sensitive to Egfr signaling and lost when signaling is inhibited. [c] This construct is a receptor activator and causes extra veins when expressed with *1348-GAL4*.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MCR
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EGF
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B
D. melanogaster 317
D. pseudo. 330
D. virilis 344
A. gambiae 287
D. melanogaster 375
D. pseudo. 388
D. virilis 402
A. gambiae 346
C
D. melanogaster 477
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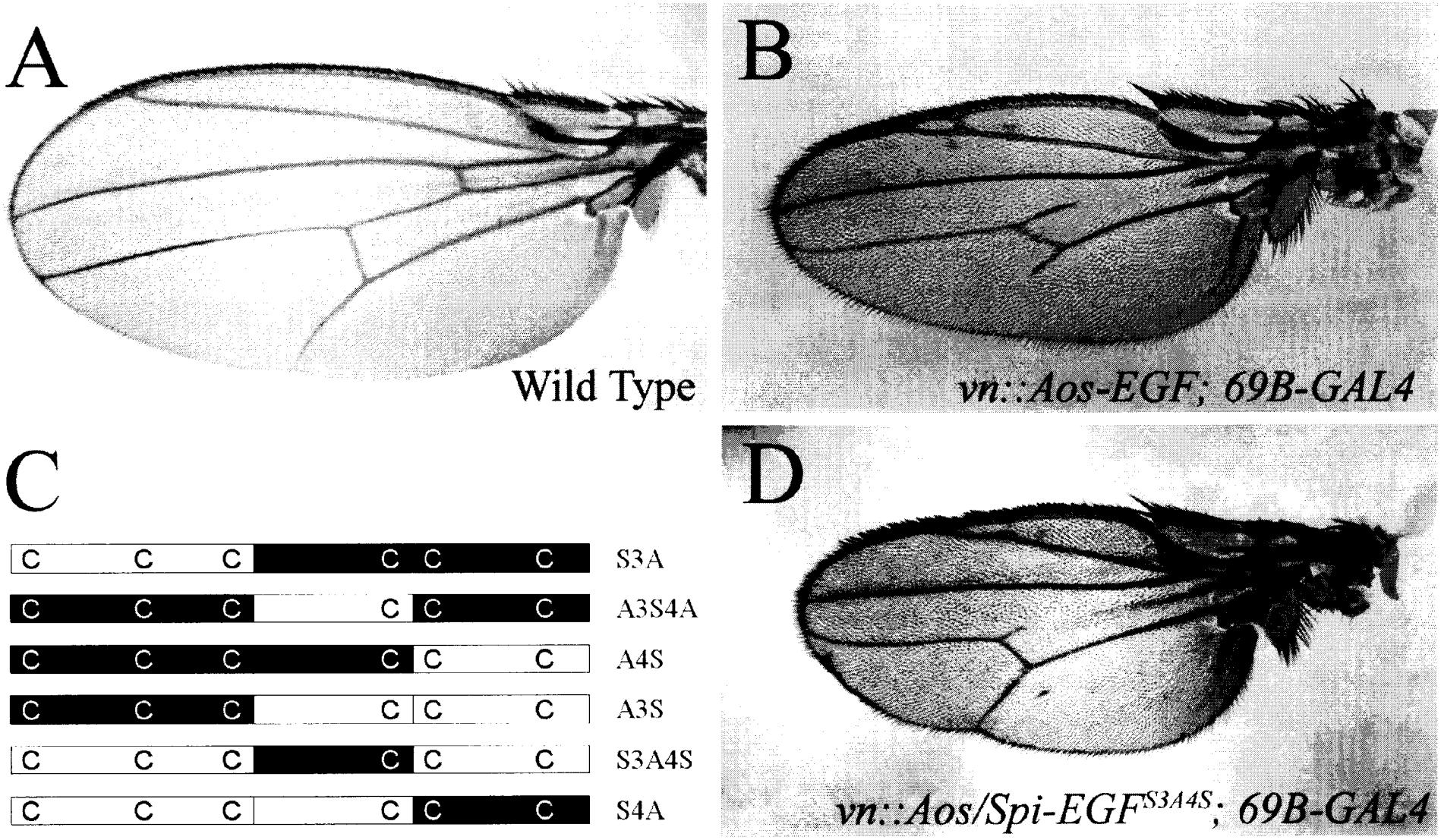
A. gambiae 527

Figure 1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Wild Type
B
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C
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C C C C C C A3S4A
C C C C C C A4S
C C C C C C A3S
C C C C C C S3A4S
C C C C C C S4A
D
vn::Aos/Spi-EGF^S3A4S; 69B-GAL4

Figure 2

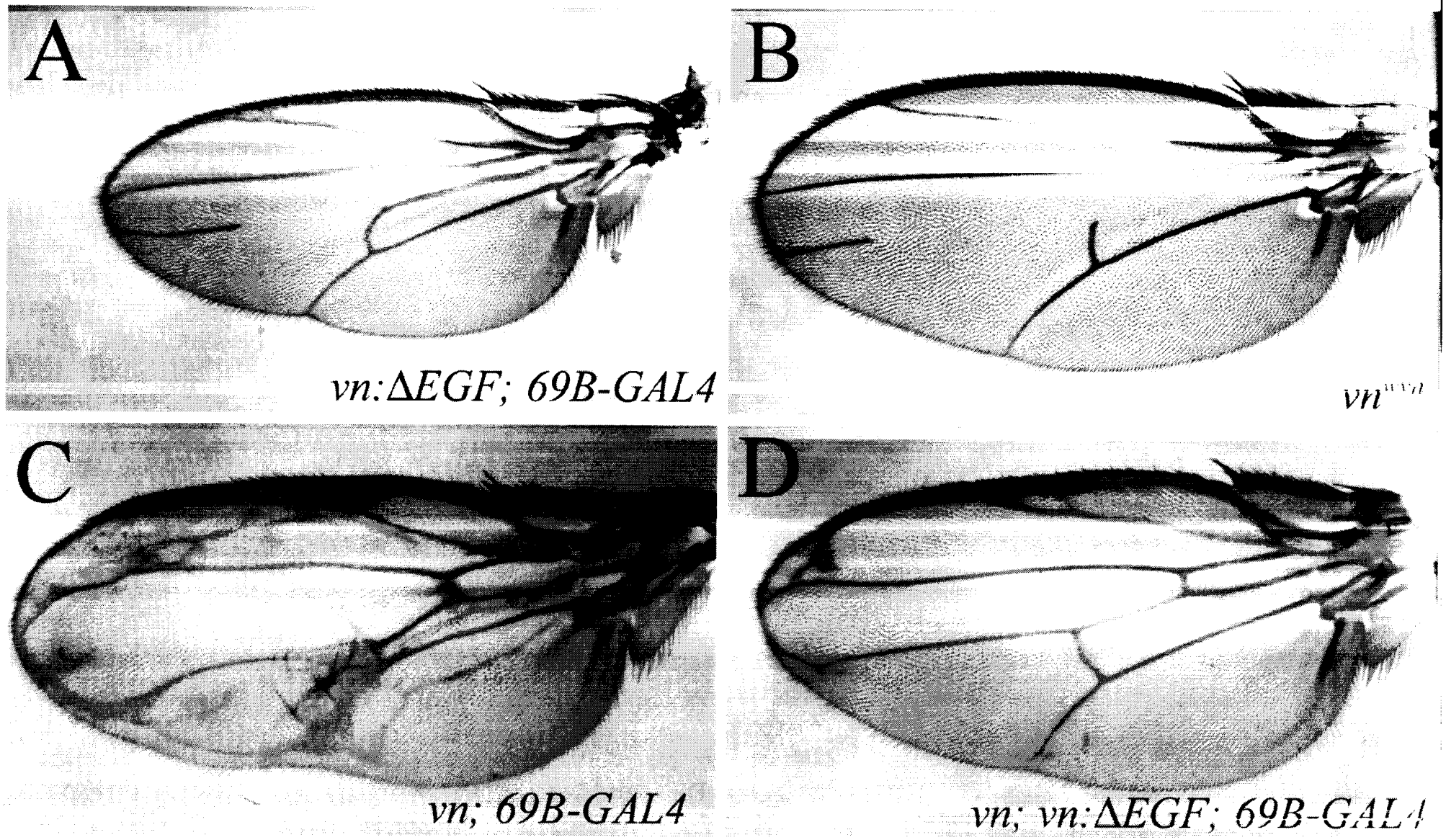
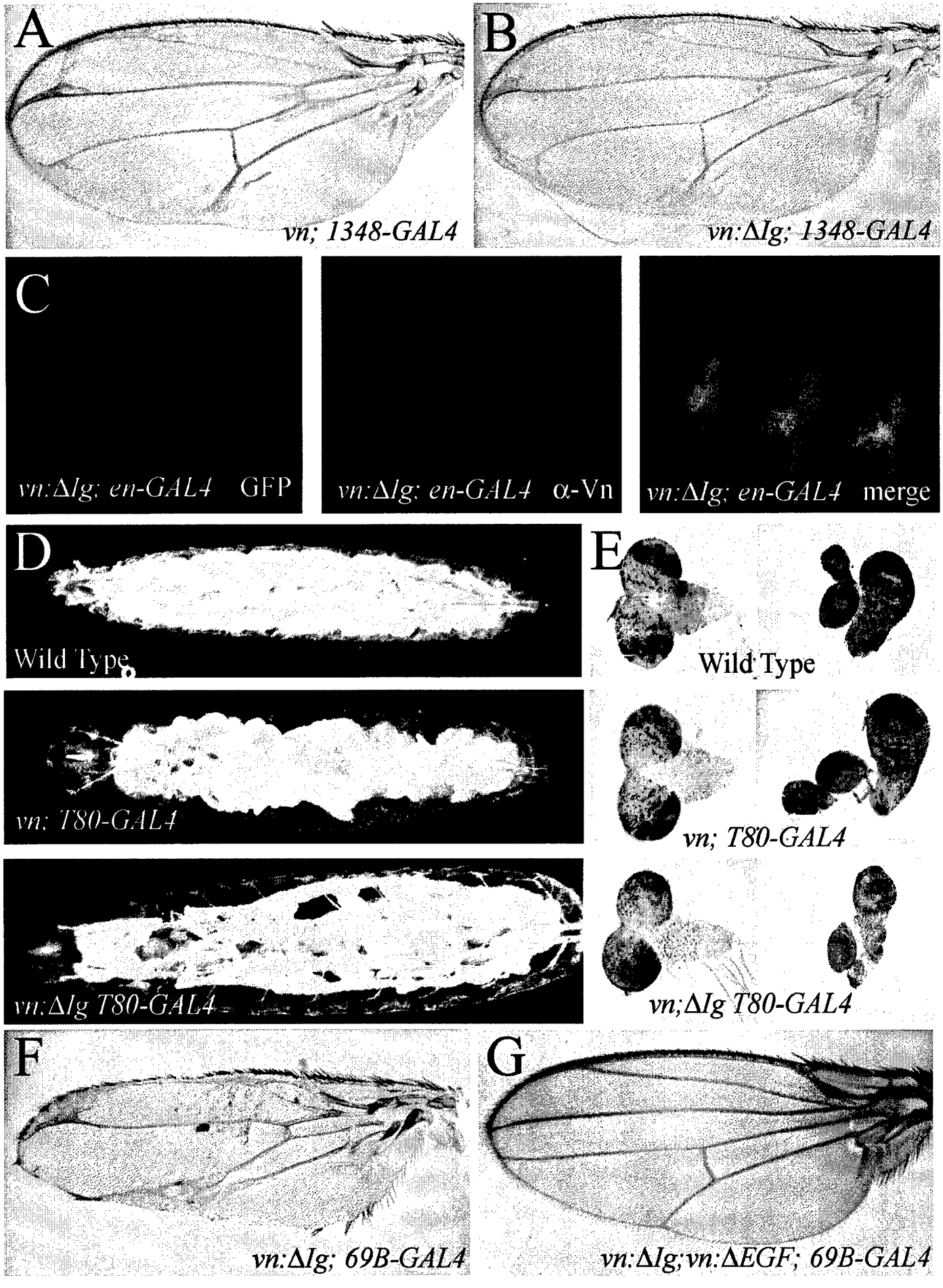


Figure 3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vn; 1348-GAL4
B
vn:ΔIg; 1348-GAL4
C
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vn:ΔIg; en-GAL4 α-Vn
vn:ΔIg; en-GAL4 merge
D
Wild Type
vn; T80-GAL4
vn:ΔIg T80-GAL4
E
Wild Type
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G
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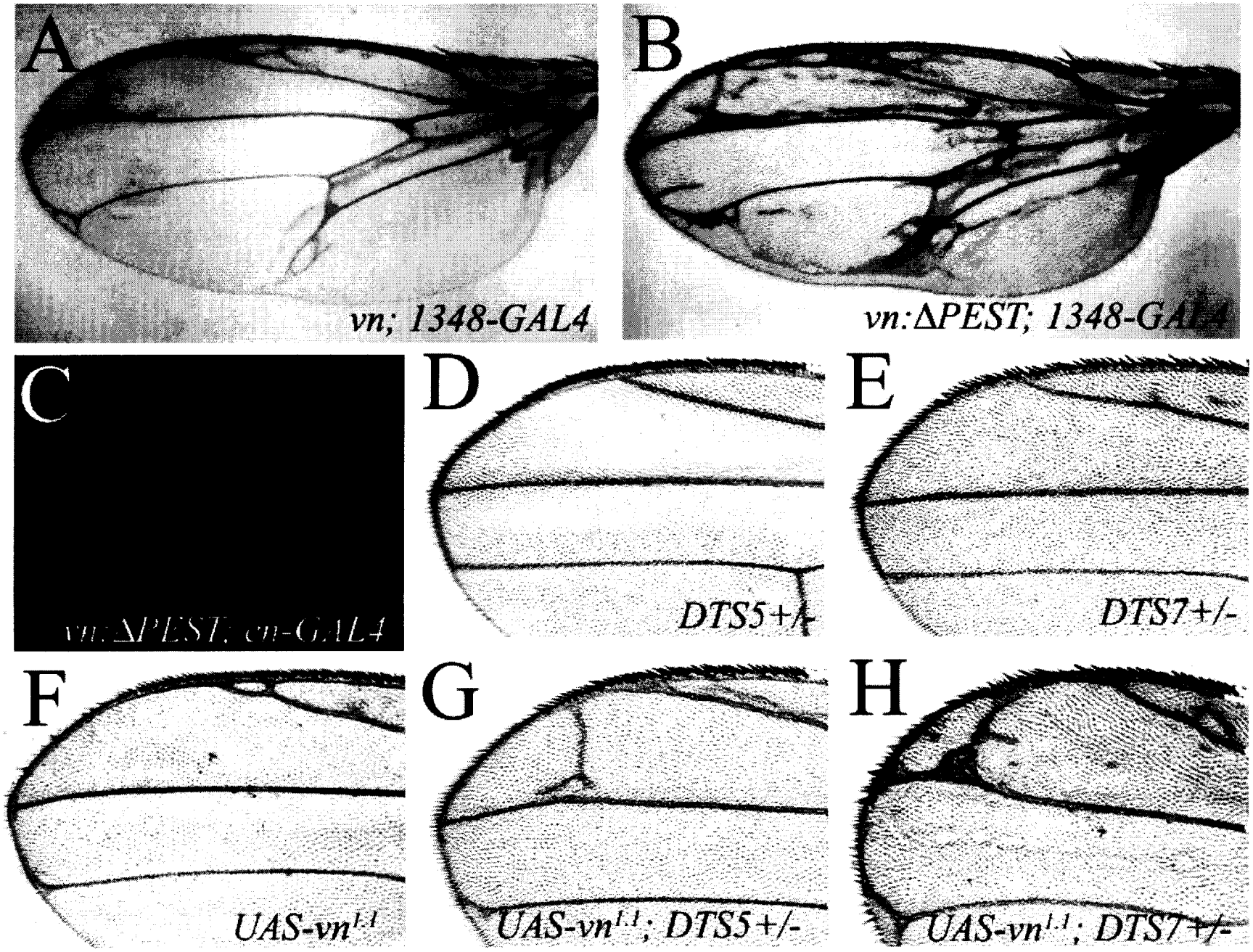
Figure 4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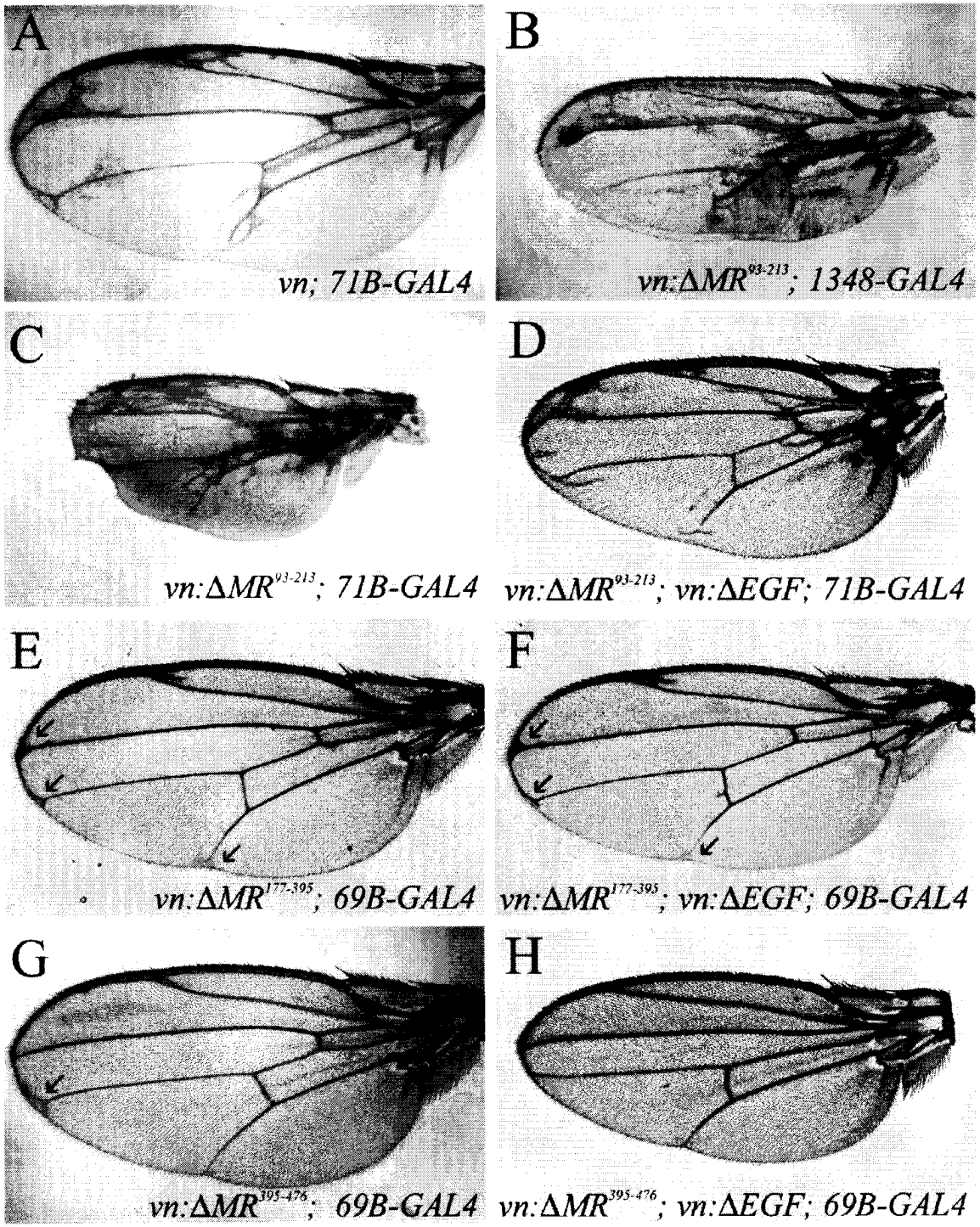
H
UAS-vn1.1; DTS7+/-

Figure 5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E
vn:ΔMR177-395; 69B-GAL4
F
vn:ΔMR177-395; vn:ΔEGF; 69B-GAL4
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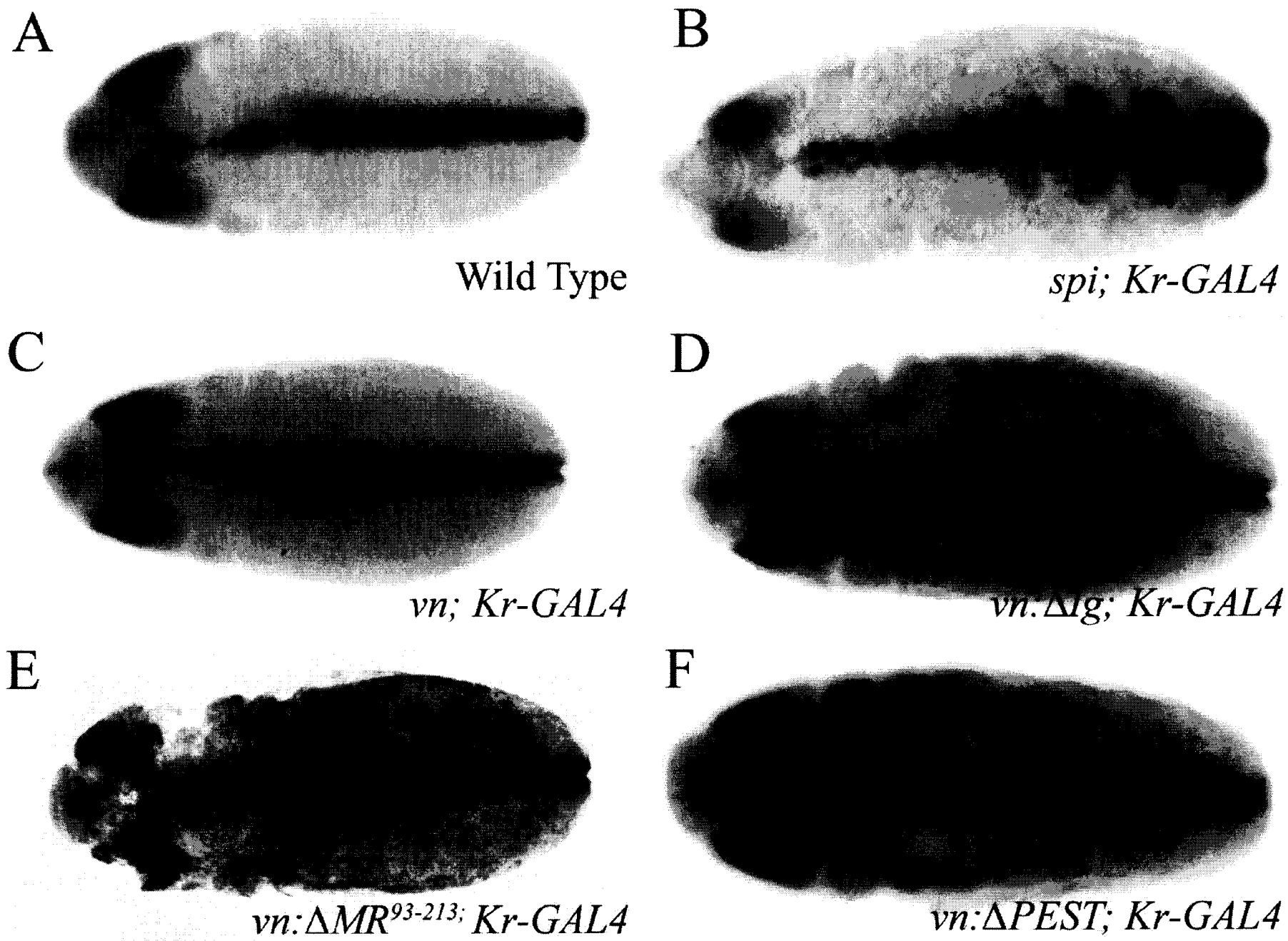
Figure 6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Wild Type
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spi; Kr-GAL4
C
vn; Kr-GAL4
D
vn:Δig; Kr-GAL4
E
vn:ΔMR93-213; Kr-GAL4
F
vn:ΔPEST; Kr-GAL4

Figure 7